Theor Appl Genet (2004) 108:982–992
DOI 10.1007/s00122-003-1516-5

ORIGINAL PAPER

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Organization of repetitive DNAs and the genomic regions carrying ribosomal RNA, *cob*, and *atp9* genes in the cucurbit mitochondrial genomes

Received: 26 August 2003 / Accepted: 25 September 2003 / Published online: 27 November 2003

Abstract Plants in the genus *Cucumis* (cucumber and melon) have the largest mitochondrial genomes known among all plants, due in part to the accumulation of repetitive DNAs of varying complexities. Recombination among these repetitive DNAs should produce highly rearranged mitochondrial genomes relative to the smaller mitochondrial genomes of related plants. We cloned and sequenced mitochondrial genomic regions near the rRNA, *atp9* and *cob* genes from cucumber, melon, squash and watermelon (all members of the Cucurbitaceae family), and compared to the previously sequenced mitochondrial genomes of *Arabidopsis thaliana* and sugar beet to study the distribution and arrangement of coding and repetitive DNAs. Cucumber and melon had regions of concentrated repetitive DNAs spread throughout the sequenced regions; few repetitive DNAs were revealed in the mitochondrial genomes of *A. thaliana*, sugar beet, squash and watermelon. Recombination among these repetitive DNAs most likely produced unique arrangements of the *rrn18* and *rrn5* genes in the genus *Cucumis*. Cucumber mitochondrial DNA had more pockets of dispersed direct and inverted repeats than melon and the other plants, and we did not reveal repetitive sequences significantly contributing to mitochondrial genome expansion in both cucumber and melon.

Communicated by R. Hagemann

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Introduction

Great differences in mitochondrial genome size and structure exist among eukaryotes. The mitochondrial genomes of animals and fungi are relatively small, for example 17 kb for humans and 55 kb for yeast, and tend to be stable in both structure and sequence (Gillham 1994). In contrast, plant mitochondrial genomes are much larger than those of animals (Gray et al. 1999), varying in size from 208 kb for white mustard (*Brassica hirta* L.) (Palmer and Herbon 1987) to over 2,400 kb for melon (*Cucumis melo* L.) (Ward et al. 1981). The increased sizes of the plant mitochondrial genomes are due in part to DNA transfers from the chloroplast (Nugent and Palmer 1988) and nucleus (Knoop et al. 1996; Notsu et al. 2002), as well as the accumulation of repetitive DNAs of varying complexities (Bendich 1979; Ward et al. 1981). Recombination among inverted repeats flips the orientation of genes; whereas recombination among direct repeats in the plant mitochondrial genomes produces smaller circular molecules of different sizes, and can result in duplications and deletions of specific regions (Fauron et al. 1995; Wolstenholme and Fauron 1995; Lilly et al. 2001; Woloszynska et al. 2001).

The mitochondrial genes encoding ribosomal RNAs (rRNAs) are differentially organized in plants, animals and fungi (Newton 1988). The rRNA genes encoding the

small and large ribosomal subunits (15S and 21S) are located distantly in yeast (*Saccharomyces cerevisae*) (Borst and Grivell 1978) and close together (12S and 16S) in mammals (Eperon et al. 1980; Van Etten et al. 1980). In most plants, the 18S and 5S rRNA genes (*rrn18* and *rrn5*) are physically close and co-transcribed (Maloney and Walbot 1990; Giegé et al. 2000), whereas the 26S rRNA gene (*rrn26*) is not physically linked to *rrn18* and *rrn5* (Huh and Gray 1982; Newton 1988; Unseld et al. 1997).

The mitochondrial genomes of *Arabidopsis thaliana* L., sugar beet (*Beta vulgaris* L.) and rice (*Oryza sativa* L.) have been sequenced (Unseld et al. 1997; Kubo et al. 2000; Notsu et al. 2002). The mitochondrial DNA of *A. thaliana* carries 57 genes among 366,924 nucleotides. Coding sequences, introns and open-reading frames (ORFs) cover approximately 28% of the genome. Direct repeats and sequences derived from the plastid or nuclear (retroelement) genomes accounted for approximately 12% of the genome, leaving the role of 60% for the mitochondrial DNA unexplained (Unseld et al. 1997). The sugar beet mitochondrial genome is similar in size and gene content to that of *Arabidopsis*, except for a few additional and missing genes relative to *Arabidopsis* (Kubo et al. 2000). Like *Arabidopsis*, 56% of the sugar beet mitochondrial genome has no obvious function (Kubo et al. 2000). The rice mitochondrial genome (490,520 bp) is the largest plant mitochondrial genome sequenced to date. Accumulation of direct repeats (26% of the entire genome), as well as transfers from the plastid and nuclear genomes (20%), explained the increased size of the rice mitochondrial genome relative to *Arabidopsis* (Notsu et al. 2002). Again, at least 50% of the sequence of the rice mitochondrial genome has no coding function and no obvious features revealing its potential function.

The Cucurbitaceae is a dicotyledonous plant family possessing species with the largest known mitochondrial genomes. The four most economically significant cucurbits, watermelon (*Citrullus lanatus* L. in Tribe Benincaseae), squash (*Cucurbita pepo* L. in Tribe Cucurbiteae), cucumber (*Cucumis sativus* L.) and melon (both in the Tribe Melothrieae), are all members of the subfamily Cucurbitoideae. Reassociation kinetics revealed sizes of 330, 800, 1,500, and 2,400 kb for watermelon, squash, cucumber and melon mitochondrial DNAs, respectively (Ward et al. 1981). These huge size differences are not due to larger mitochondria (Bendich and Gauriloff 1984), duplication of the entire genome (Havey et al. 1998) or highly repetitive DNAs (Ward et al. 1981), but are associated with the accumulation of middle repetitive DNAs (Ward et al. 1981). In cucumber, short repetitive DNA motifs (30 to 53 bp) of varying degeneracies accounted for approximately 13% of the cucumber mitochondrial genome (Lilly and Havey 2001). Recombination among these repetitive DNAs should produce unique arrangements of conserved coding regions. We studied mitochondrial genome organization and expansion in the cucurbits by assembling and sequencing the mitochondrial contigs carrying the *atp9*, *cob* and rRNA genes. Our analyses reveal unique arrangements of the *rrn18* and *rrn5* genes in *Cucumis*, as well as differences in the amounts and distributions of repetitive DNAs as compared to fully sequenced plant mitochondrial genomes of smaller sizes.

Materials and methods

Plant materials and mitochondrial DNA libraries

Seeds from cultivars of cucumber 'Calypso' (Asgrow), melon 'Iroquois' (Asgrow), squash 'Black Beauty' (Hollar Seeds) and watermelon 'Dixielee' (Hollar Seeds) were planted in vermiculite, and germinated in the dark at 28–30° for 4 to 7 days. Mitochondrial DNA was extracted and large-insert (8 to 15 kb) libraries were constructed in plasmids as described by Lilly and Havey (2001). DNA-blot hybridizations using total plant DNA were performed to estimate relative copy numbers of major mitochondrial genes among the major cultivated cucurbits. Clones of *atp6* and *atp9* (Dewey et al. 1985), *cob* (Dawson et al. 1984), *coxI* (Isaac et al. 1985) and *coxIII* (Hiesel et al. 1987) were hybridized to *Bam*HI, *Bgl*II, *Bst*EII, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II, *Xba*I and *Xho*I digests (Havey et al. 1998). Primers [18S_SpecF (5′-TTGGCAGGGCGTACTAAACC) for positions 3,694 to 3,713 and 18S_SpecR (5′-GAGAAATGCAACCGAAGTTC) for positions 3901 to 3925] were designed to amplify a 231-bp fragment from the cucumber *rrn18* clone (GenBank Acc. AY258278) corresponding to the V7 region specific to the plant mitochondrial 18S rRNA (Neefs et al. 1990), eliminating cross-hybridization with the chloroplast 16S rRNA. The cucumber amplicon was TA cloned and hybridized to *Bam*HI, *Eco*RI and *Hin*dIII digests of the cucurbit DNAs listed above.

Clones of *cob*, *atp9* and *rrn18* were chosen to screen mitochondrial DNA libraries for their respective genomic regions. Bacteria colonies were spotted onto the Zetaprobe GT nylon membrane (BioRad) using a replicator, and grown overnight at 37°C on solid LB medium with ampicillin (100 μg/ml). Bacterial colonies were removed, membranes washed and DNA fixed to nylon membranes (Nizetic et al. 1991; Lilly and Havey 2001). Membranes were hybridized with *atp9*, *cob* and *rrn18*, washed twice (0.1×SSC with 0.2% SDS) for 30 min at 65°C, and subjected to autoradiography. Colonies showing the strong hybridization signal were selected and plasmids were isolated (Qiagen, Valencia, California).

Sequencing and analyses of mitochondrial DNA Clones

Clones were sequenced using random transposon insertions (EZ::Kn2 system, Epicentre, Madison, Wisconsin) and cycle-sequencing reactions (Lilly and Havey 2001). Sequences were edited, trimmed, and contigs aligned using Sequencer 3.1.2 (Genecodes, Ann Arbor, Michigan). Sequences of cucumber *cob* (AF288044), *atp9* (AF288043), *rrn18* (AY258278) and the watermelon *atp9* (AF288042) were previously described (Lilly and Havey 2001; Bartoszewski et al., in press). Computer analyses were conducted both within individual sequences and among the cucurbit *atp9* and *cob* genomic regions. Similarity searches were conducted using BLASTN and TBLASTX algorithms (Altschul et al. 1997) to public databases. Putative open reading frames (ORFs) of at least 100 amino acids were identified using Sequencer 3.1.2 (Genecodes) and the FRAMES command in the Genetic Computer Group (GCG) Wisconsin Package version 10.3. ORFs showing no homologies to database sequences were designed as orfX, with X indicating the putative number of amino acids. Transfer (t) RNAs were identified using the tRNAscan program (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/) (Lowe and Eddy 1997). Homologies at a minimum of 100 bp and showing at least 70% identity to non-coding areas of other mitochondrial sequences were

Table 1 Plants evaluated for orientation of the *rrn18* and *rrn5* mitochondrial genes

No.	Plant	Source[1]	*rrn18* and *rrn5* Organization
1	*C. africanus*	PI 542127	*rrn5–rrn18*
2	*Cucumis sagittatus*	PI 282441	Unlinked
3	*Cucumis anguria var. anguria*	Ames 23536	Unlinked
4	*C. anguria var. longaculeatus*	PI 249897	Unlinked
5	*Cucumis callosus*	PI 532839	Unlinked
6	*Cucumis dipsaceus*	PI 390450	Unlinked
7	*C. melo* var. *chito*	PI 164327	Unlinked
8	*Cucumis pulsulatus*	PI 532673	Unlinked
9	*C. sativus* var. *hardwickii*	PI 462369	*rrn5–rrn18*
10	*Cucumis myriocarpus* subsp. *leptodermis*	PI 374152	Unlinked
11	*Cucumis meeusei*	PI 376068	Unlinked
12	*C. melo* subsp. *Agrestis*	PI 614531	Unlinked
13	*C. melo* var.*cantalupensis*	PI 266948	Unlinked
14	*C. melo* var. *conomon*	PI 532830	Unlinked
15	*C. melo* var. *flexuosus*	PI 279366	Unlinked
16	*C. melo* var. *inodorus*	PI 420152	Unlinked
17	*Cucumis membranifolius*	PI 273650	Unlinked
18	*Cucumis myriocarpus*	PI 299568	Unlinked
19	*C. myriocarpus* subsp. *myriocarpus*	Ames 23542	Unlinked
20	*Cucumis prophetatum*	PI 193967	Unlinked
21	*C. sativus* var. *sikkimensis*	PI 504568	*rrn5–rrn18*
22	*C. sativus* var. *xishuangbannanesis*	PI 618931	*rrn5–rrn18*
23	*Cucumis trigonus*	Ames 24297	Unlinked
24	*C. zeyheri*	PI 364472	*rrn5–rrn18*
25	*C. lanatus*	PI269341	*rrn18–rrn5*
26	*C. pepo*	Guard	*rrn18–rrn5*
27	*C. pepo*	Dark Green Zucchini	*rrn18–rrn5*
28	*C. pepo*	Acorn	*rrn18–rrn5*
29	*C. moschata*		*rrn18–rrn5*
30	*C. melo* var. *melo*	Iroquois	Unlinked
31	*C. melo* var. *melo*	PI357775	Unlinked
32	*Lycopersicon esculentum*	Rutgers	*rrn18–rrn5*
33	*Solanum tuberosum*	Kennebec	*rrn18–rrn5*
34	*Daucus carota*	Savory 1227	*rrn18–rrn5*
35	*Brassica rapa*		*rrn18–rrn5*
36	*Gycine max*	216.2	*rrn18–rrn5*
37	*O. sativa*		*rrn18–rrn5*
38	*Zea mays*		*rrn18–rrn5*
39	*C. sativus*	Line B	*rrn5–rrn18*
40	*A. thaliana*	Columbia	*rrn18–rrn5*

[1] Seed sources for numbers 1 through 25 were plant introductions (PIs) provided by the USDA National Plant Germplasm System

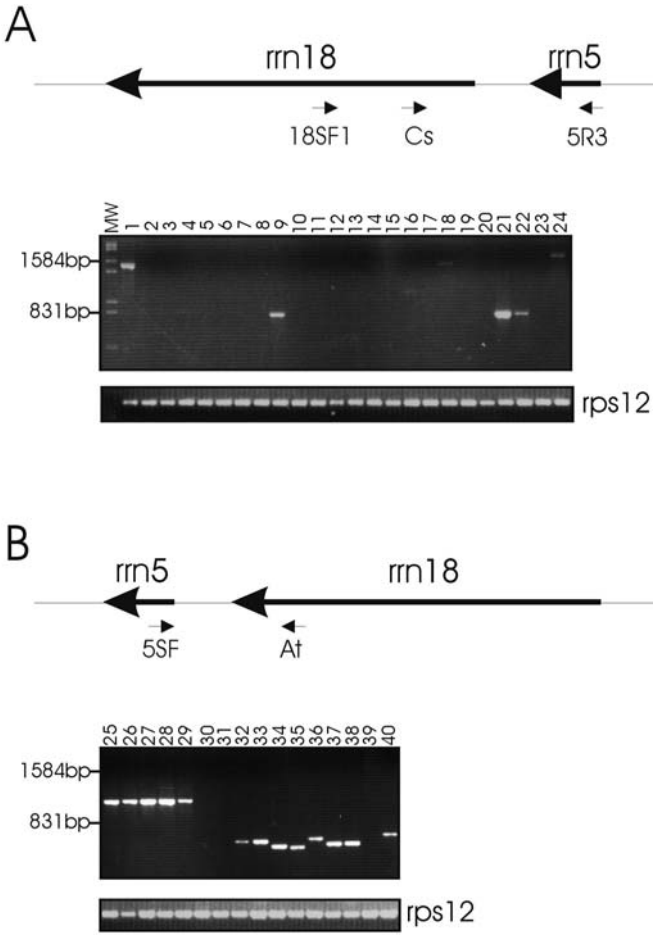

Fig. 1 Organization of *rrn5* and *rrn18* genes in cucumber (**A**) and *A. thaliana* (**B**). Primer positions and names are shown *below lines* (not drawn to scale). Gels show amplicons from accessions listed in Table 1 using primers specific for the *rrn5* and *rrn18* gene order of cucumber (**A**) and *A. thaliana* (**B**). No bands were amplified from accessions 1 to 24 using the *Arabidopsis* primers and from accessions 25 to 38 and 40 using cucumber primers (gels not shown). MWt is the molecular weight standard. Positive control for amplification was a 320-bp *rps12* fragment shown below each gel

compiled by extracting BLASTN output. Repeatmasker (http://ftp.genome.washington.edu/RM/RepeatMasker.html; Smit and Green, unpublished) was used to search for repetitive elements against RepBase version 07/07/01 (Jurka 2000). We used Tandem Repeat Finder (Benson 1999) and REPuter (Kurtz and Schleiermacher 1999) to search for genomic sequences near the cucurbit *atp9* and *cob* genes for direct tandem repeats, allowing for 3-bp maximum distance between repeats and requiring no minimum repeat length. We also searched for dispersed direct repeats and inverted repeats longer than 25 bp allowing up to three mismatches and no maximum distance between repeats. Segmental self-comparison percent identity plots were generated by PipMaker (Schwartz et al. 2000).

Inverted-repeat sequences were revealed using the FINDMITE program as described by Tu (2001). We created a database containing the complete mitochondrial sequences of *A. thaliana* (Unseld et al. 1997) and sugar beet (Kubo et al. 2000), all publicly available cucumber mitochondrial sequences (Lilly and Havey 2001; Lilly et al. 2001; Bartoszewski et al., in press), all GenBank mitochondrial sequences of the cucurbits (watermelon accession X04130 and AF288044) and sequences generated in this study, yielding a total of 265.1 kb (17.6% of the mitochondrial genome) for cucumber, 20.1 kb (0.6%) for melon, 14.3 kb (1.8%) for squash and 24.3 kb (7.3%) for watermelon. These sequence databases were searched for direct repeats of NN, NNN, NNNN and NNNNNNNN where N is A, T, G or C; terminal inverted repeat (TIR) length of 11 with 1 mismatch was allowed; filtering of A/T, C/G and AT/TA; no TIR 2 base-pair composition filtering, and lengths of 30 to 700 bp.

PCR reactions

Primers were designed to reveal the organization of *rrn5* and *rrn18* among *Cucumis* species (Table 1) based on the *A. thaliana* (primer set At and 5SF) and cucumber (primer set Cs and 5SR3) gene orders (Fig. 1) [At: (5′-TTCGGATTGTTCTCTGCAACT), 5SR3: (5′-AGATTGTTCGGGAGACATGGT), Cs: (5′-TTCACTTT-CAACCCGATTCAC) and 5SF: (5′-ACCATGTCTCCCGAA-

CAATCT), which was complementary to the 5SR3 primer]. As controls, PCR reactions were completed using primers amplifying from *rps12* (rps12F1: TGGGTTTTTCTGCACCATATT, and rps12R1: 5′-GCGCACGGACCGTACTCGAGC); and *rrn18* (18S_179F 5′-GGTGTGACGGGCGGTATGTA and 18S_1841R 5′-AGTAGTACGCCCGGTTCACTAGGT). DNA was extracted from the accessions listed in Table 1 using the DNeasy kit (Qiagen California, USA). PCR was performed using Ex*Taq* according to the manufacturer's instruction (Takara Shuzo Co., Japan), and cycling conditions of 94°C for 2 min (initial denaturation), 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 3 min and extension time of 68°C for 10 min. PCR products were electrophoresed and bands cut out and purified using the Qiaquick gel purification kit (Qiagen). Purified bands were cloned using the TOPO cloning system (Invitrogen, USA) and sequenced as described earlier. Long distance PCR, as described by Lilly et al. (2001), was used to verify insertion of the *rrn23* chloroplast region into the melon mitochondrial genome (accession AY305264) using primers mel_cp23SF (5′-TACCATAGAGGCCAAGGATAGACA) and mel_atp9R (5'-GTTTAGCCAATGATGGATTTCG).

Northern blot and RT-PCR

For RNA gel-blots, total cucumber RNA was extracted from young cucumber leaves as described by Chomczynski and Sacchi (1987). Twenty micrograms of RNA was fractionated on the formaldehyde gel, blotted, hybridized and subjected to autoradiography (Bartoszewski et al., in press). The *rrn5* gene-specific fragment was amplified using primers 5SF and 5SR1 (CTTTTGGTCCTTTG-GACAGGT) from the *A. thaliana* cosmid carrying *rrn5* (Klein et al. 1994) using conditions described earlier. RT-PCR was completed using 1.5 μg of total cucumber RNA treated with DNaseI (Promega, Wisconsin) and the 18S RNA specific primer (Fig. 1) 18S-F1 (GTGTGTGGCCCAGCCCATAA). Reverse transcription reaction was used as a template (1 μl) for the PCR reaction with primer set Cs and 5SR3 to amplify a 812-bp intergenic fragment as described above. RT-PCR control reactions used water instead of reverse transcriptase.

Results and discussion

Organization of *rrn18* and *rrn5* in *Cucumis* species

We cloned and sequenced the cucumber mitochondrial DNA region carrying *rrn18* and *rrn5* (GenBank Acc. AY258278), and discovered that these genes have a different orientation (Fig. 1) compared to *A. thaliana*, sugar beet and rice (Unseld et al. 1997; Kubo et al. 2000; Notsu et al. 2002). We determined the organization of *rrn18* and *rrn5* in wild and cultivated *Cucumis* species by designing primers amplifying across the intergenic spacer between *rrn5* and *rrn18*. The cucumber arrangement was amplified from all accessions of cucumber, including *C. sativus* var. *hardwickii*, *C. sativus* var. *sikkimensis* and *C. sativus* var. *xishuangbannanesis* (Table 1). Amplicons from *Cucumis africanus* and *Cucumis zeyherii* were larger and in the same orientation as cucumber (Fig. 1A). None of the other *Cucumis* species, including melon (*C. melo*) and other plants (Table 1), produced amplicons using primers specific for the cucumber orientation (Fig. 1A). Using primers corresponding to the *Arabidopsis* arrangement (Fig. 1B), amplicons were observed for *Cucurbita* and *Citrullus*, as well as for tomato, potato, carrot, corn and rice (Fig. 1B). The watermelon *rrn18–rrn5* intergenic spacer was longer than the other plants (Fig. 1B). All amplicons were cloned and sequenced to verify their homologies (Table 2).

None of the *rrn18–rrn5* primer sets amplified across the intergenic region from melon, the subspecies of melon and some wild African *Cucumis* species (Table 1). DNA-gel blots hybridized with *rrn18* revealed a single strong band for melon (autoradiogram not shown). However differently sized fragments were revealed after hybridization of *rrn5* (4 to 5 bands depending on the restriction enzymes; autoradiogram not shown). We isolated and sequenced the *rrn18* homologous region from melon mitochondrial DNA and found that no *rrn5* gene exists

Table 2 Sequence size and characteristics of cucurbit mitochondrial genomic regions carrying the *atp9*, *cob* and rRNA genes

Genomic region	Cucurbit	Size (bp)	Genebank accession	%GC
atp9	Watermelon	11,863	AF288042[1]	45.9
	Squash	9,147	AY305266	44.3
	Cucumber	13,276	AF288043[1]	45.0
	Melon	10,403	AY305264	42.8
cob	Watermelon	6,491	AY305267	44.8
	Squash	6,898	AY305268	45.5
	Cucumber	16,027	AF288044[1]	44.2
	Melon	12,006	AY305265	44.0
rrn18–rrn5	Watermelon	8,901	AY357215	46.8
	Cucumber	5,219	AY258278[2]	46.0
	Melon	10,670	AY357214	46.0
rrn18–rrn5 intergenic spacer	*C. sativus* var. *sikkimensis*	812	AY357206	50.4
	C. africanus	1,460	AY357205	40.1
	C. zeyherii	1,641	AY357207	39.3
	C. moschata	939	AY357208	50.9
	C. pepo	939	AY357209	50.9

[1] Sequences were updated from those described by Lilly and Havey (2001)
[2] Sequences described by Bartoszewski et al. (in press)

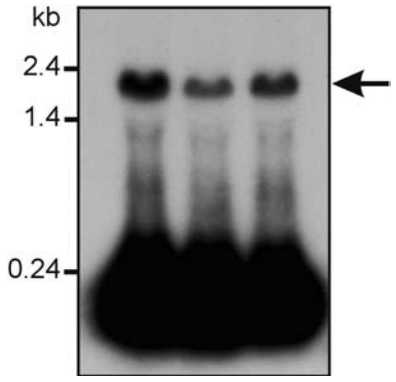

Fig. 2 RNA-gel-blot hybridized with *rrn5* probe showing co-transcription of *rrn18* and *rrn5* (fragment indicated by the *arrow*) in cucumber accessions line B, MSC16 and Calypso (*lane orders from left to right*). The lower fragment corresponds to the 5S rRNA. Size marker in kilobases (kb) shown on left side

within 2.18 kb of *rrn18* (Genbank accession AY357214), demonstrating that the *rrn18* and *rrn5* genes are not physically close in melon. We isolated four clones carrying *rrn5* from the melon mitochondrial library and sequenced outwards using the *rrn5* specific primers (Genbank Acc. AY357210 through AY357213); no *rrn18* was revealed within 720 bp on either side of *rrn5*. We also observed no amplicons using the primers specific to *rrn18* and *rrn5*, and long-distance PCR with 15-min extension times. In the past we have used these PCR conditions to amplify from the mitochondrial DNA fragments greater than 13.0 kb (Lilly et al. 2001), indicating that no *rrn18* lies close to *rrn5* in melon.

Co-transcription of *rrn5* and *rrn18* in cucumber

We probed cucumber RNA-gel blots with the mitochondrial-specific *rrn5* to reveal two fragments; one smaller strong band corresponded to *rrn5* and one larger lighter band corresponded in size to co-transcribed *rrn5* and *rrn18* (Fig. 2). We performed RT-PCR using primers specific for *rrn18* to synthesize the first cDNA strand, followed by primers specific for *rrn5* and *rrn18* to amplify the expected 812-bp fragment from cucumber mitochondrial RNA, but not control reactions (gel not shown). This verifies that the uniquely arranged *rrn5* and *rrn18* genes in cucumber are co-transcribed.

Sequence analyses of cucurbit *atp9* and *cob* mitochondrial regions

DNA blot hybridizations with *cob*, *coxI*, *coxIII* and *atp6* revealed one or two fragments across numerous restriction enzymes, indicating that these genes exist as single copies in the cucurbit mitochondrial genomes, except for two *atp9* fragments in watermelon and two *atp6* fragments in melon (autoradiograms not shown). We chose to clone and sequence mitochondrial regions carrying the *atp9* and *cob* genes from cucumber, melon, squash and watermelon (Table 2). In spite of the huge differences in mitochondrial genome sizes among the cucurbits, sequence analyses revealed that the mean percent GC contents of cucumber (44.6±0.6 as estimated from mitochondrial regions carrying *atp9* and *cob*), melon (43.4±0.8), squash (44.9±0.8) and watermelon (45.4±0.8) were similar to those of *A. thaliana* (44.8), sugar beet (43.9) and rice (43.8) (Table 2). As expected, the smaller mitochondrial genome of watermelon possessed greater gene densities than the larger mitochondrial genomes of squash, cucumber or melon (Fig. 3, Table 3). The watermelon *atp9* region carried coding regions for *trnG*, *trnQ*, *nad9*, *nad5-exon1*, *nad5-exon2* and an *sdh3* pseudogene (as defined by premature stop codons). The mitochondrial genomes of watermelon and squash shared a region of high similarity adjacent to the *atp9* gene carrying *nad5-exon1* and *nad5-exon2*. As we previously reported (Lilly et al. 2001), the *atp6* gene lies close to *atp9* in cucumber. In melon, the mitochondrial region carrying *atp9* also possessed a pseudo-*atp6* gene. In contrast to watermelon and squash, the *atp9* regions of cucumber and melon only possessed short regions of high similarities with these cucurbits and other sequenced mitochondrial genomes (Fig. 3A, Table 3). Melon carried two ORFs of unknown origin and expression. Short regions of high similarities to chloroplast DNA (green in Fig. 3A) were found in watermelon and cucumber; however, a much larger (3,656 bp) chloroplast transfer was revealed in melon (Fig. 3A, Table 3). This melon region showed similarity to chloroplasts *rrn23*, *rrn4.5* and *rrn5*. The largest reported chloroplast insertions into plant mitochondrial genomes are 930 bp in *Arabidopsis* (Unseld et al. 1997), 3,366 bp in sugar beet (Kubo et al. 2000) and 6,653 bp in rice (Notsu et al. 2002), but none showed similarity to the *rrn23*, *rrn4.5* and *rrn5* cluster. The long-distance PCR with primers specific for *atp9* and the chloroplast 23S rRNA amplified the expected product of 4,856 bp from melon, but not cucumber; and confirmed that this insertion was not a cloning artifact.

The watermelon and squash *cob* regions were more gene-dense as compared to this region in cucumber and melon (Fig. 3B). Watermelon possesses a pseudo-*atp9* in front of the *rpl5–rps14–cob* cluster. Both squash and watermelon possessed the *rpl5* coding region adjacent to *cob* with a pseudo-*rps14* (Fig. 3B, Table 3). This arrangement of the *rpl5–pseudo–rps14–cob* is conserved in both *Arabidopsis* and potato (Aubert et al. 1992; Quiñones et al. 1996); other plants (such as *Pisum sativum* and *Brassica napus*) possess the *rpl5–rps14–cob* cluster with a functional *rps14*, and these genes are co-transcribed (Ye et al. 1996; Hoffmann et al. 1999). Regions of strong sequence similarities in the intergenic regions were evident in squash and watermelon, with a specific region of high similarity adjacent to *cob* present in all four cucurbits (Fig. 3B).

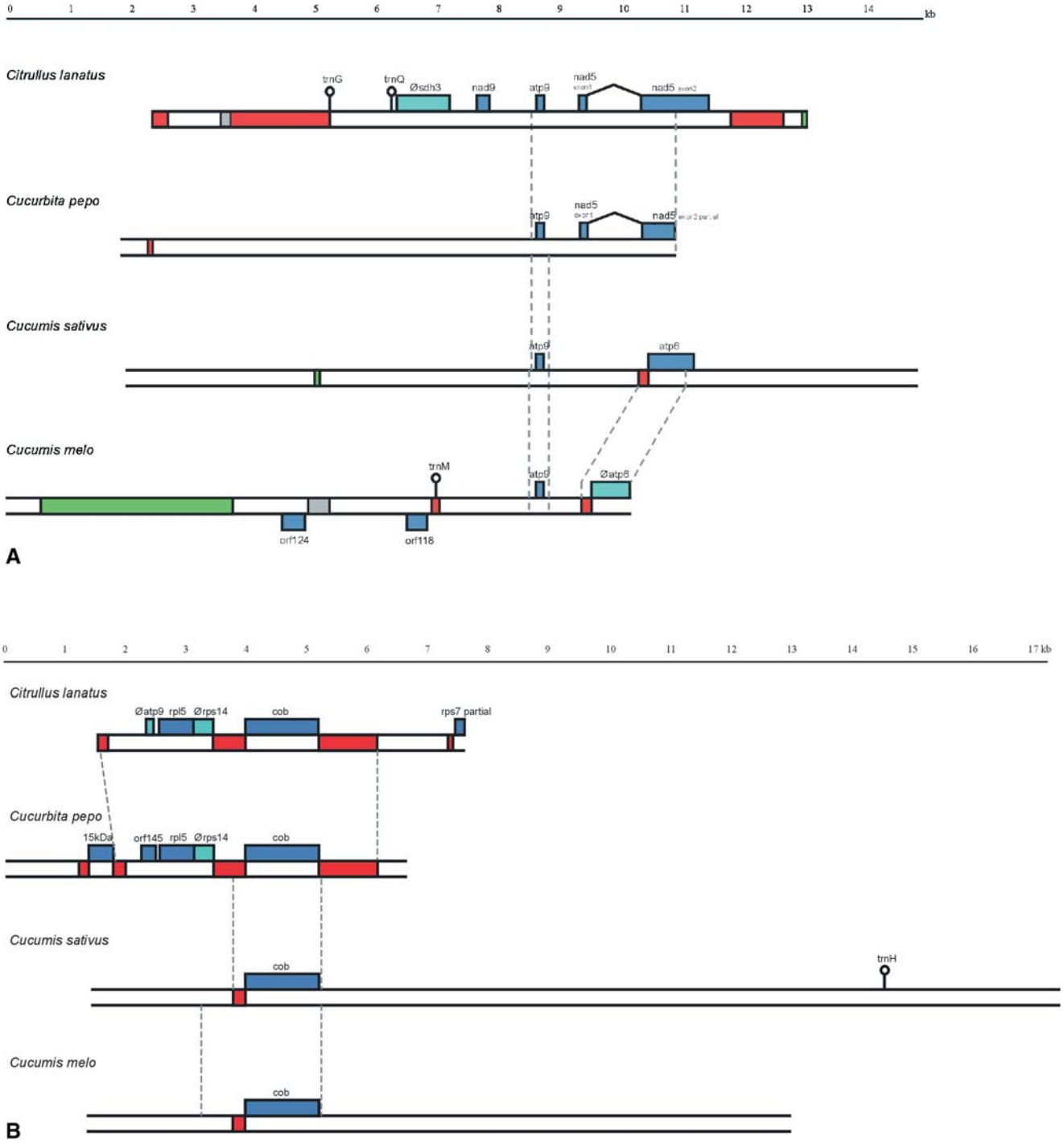

Fig. 3 Schematic diagrams showing gene organization of the *atp9* (**A**) and *cob* (**B**) regions of the mitochondrial DNA of watermelon, squash, cucumber and melon. *Bars* represent the mitochondrial sequences. *Boxes* above or below the bars indicate genes or open-reading frames (ORFs) located on the forward or reverse strands, respectively. *Blue boxes* show regions homologous to known genes in another plants or ORFs of 100 amino acids or more. *Red boxes* show areas with high similarities with other mitochondrial sequences of greater than 100 basepairs (bp) and at least 70% identity. *Cyan boxes* indicate pseudogenes. *Green boxes* indicate similarities with chloroplast sequences. Grey boxes indicate similarities to retroelements. Transfer RNAs are represented by cloverleaf structure. *Vertical lines* show regions of high sequence similarities among the cucurbits

Table 3 Regions of high similarities surrounding the *atp9* and *cob* genes in the major cucurbit crops as compared with other plant mitochondrial DNAs (minimum of 100-bp long with 70% identity)

Genomic region	Cucurbit	Size	Position	Similarity[1]
atp9	Watermelon	610	1–611	*Beta vulgaris* non-coding mitochondrial DNA
		201	1,237–1,438	*A. thaliana* retroelement ATCOPIA7I
		1,543	1,450–2,993	*B. vulgaris* non-coding mitochondrial DNA
		732	9,682–10,414	*B. vulgaris* non-coding mitochondrial DNA
		80	11,783–11,863	*A. thaliana* chloroplast *rpoB* RNA polymerase
	Squash	107	443–550	*Lotus corniculatus* non-coding mitochondrial DNA
	Cucumber	96	3,127–3,223	*A. thaliana* non-coding chloroplast DNA
		162	8,593–8,755	*B. vulgaris* non-coding mitochondrial DNA surrounding *atp6*
	Melon	3,656	568–4,224	*A. thaliana* chloroplast 23S RNA operon
		409	4,789–5,198	*A. thaliana* retroelement ATCOPIA32
		216	6,867–7,083	*B. vulgaris* non-coding mitochondrial DNA
cob	Watermelon	256	1–257	*A. thaliana nad5* intron
		301	1,924–2,225	*A. thaliana* non-coding mitochondrial DNA
		957	3,768–4,725	*P. sativum* non-coding mitochondrial DNA
		136	5,925–6,061	*A. thaliana* non-coding mitochondrial DNA
	Squash	221	1,206–1,427	*A. thaliana* non-coding mitochondrial DNA
		227	1,845–2,072	*A. thaliana nad5* intron
		608	3,546–4,154	*A. thaliana* non-coding mitochondrial DNA
		1,077	5,334–6,411	*Vicia faba* non-coding mitochondrial DNA
	Cucumber	269	2,311–2,580	*V. faba* non-coding mitochondrial DNA

[1] Similarities with highest significance are listed

Table 4 Tandem repeats identified in the *atp9* and *cob* mitochondrial genomic regions among the cucurbits

Species	Name	Tandem repeat consensus	Length	Copy number[1]	Percent matches
Cucumber	Cs_TR01	GTAGGACCATCCGTAATTAACAGAA	25	3.3	71
	Cs_TR02	TCGCATATTTCATTGCGCCT	20	2.0	100
	Cs_TR03	AAGGAGAGGAGTGGAACTGCTCGACTGT	28	2.4	100
	Cs_TR04	CTACTTCGGTTAATTACGGATGGTC	25	2.2	96
	Cs_TR05	GGATGGTCCTACGGATAAATCACCTGGACCTTTCTTTG-GCCCATAT	46	1.9	100
	Cs_TR06	TCCCTCTCCCTACGGTCGAGCTATTTCA	28	3.4	100
	Cs_TR07	CTGCGGACGTAGGA	14	2.1	100
	Cs_TR08	GTCCTACGTCCGCAGGGCCAAAGAAAGGTCCAGG	34	2.0	100
	Cs_TR09	GTAGGACCATCCGTAATTAACCGAA	25	2.0	100
	Cs_TR10	GAAGGAGGACCATCCATATGGAGG	24	1.9	90
Melon	Cm_TR01	TGGTTTTCTCATGTTGTCAAAGAGTTGAACAA	32	2.1	94
	Cm_TR02	CTCCTTATCAGTCGAGCTGCCTCATTGA	28	3.5	75
	Cm_TR03	CCTTATCAGTCGACTTTCTGCGAA	24	3.1	70
	Cm_TR04	CCTTATCAGTCGAGCTGCCTCATTGACTCCTTATCAG-TCGACTTTCTGCGAA	55	2.0	100
Squash	Cp_TR01	ATCACAGCACTTGCTTTCCCTTTTCTATCG	29	3.0	78
	Cp_TR02	AATGAG	6	6.0	100
	Cp_TR03	GGCACCAGCTTCAGTGGGTACATCCGCGGGCCCAGCC-CCCGAAAATAACAATAACCCAGATGTGATTGCAGCAGG	75	3.0	100
Watermelon	None				

[1] Copy number is the average number of tandem repeats

Types and distributions of repetitive DNAs

There were clear differences in the distributions of repetitive DNAs in the cucurbit mitochondrial DNAs. Both Dot Plots and PipMaker (Fig. 4) revealed few repetitive DNAs in the mitochondrial genomes of *A. thaliana*, squash and watermelon. Cucumber and melon had regions of concentrated repetitive DNAs spread throughout the sequenced regions. However, cucumber possessed more distinct regions of repetitive DNAs than melon, even though the cucumber mitochondrial DNA is 35% smaller than melon. We searched genomic sequences near the cucurbit *atp9* and *cob* genes for direct tandem repeats, dispersed direct repeats and inverted repeats. Relatively few tandem repeats were identified among plants with large differences in mitochondrial-DNA sizes. Ten tandem repeats were identified in cucumber, three in squash and four in melon; no tandem repeats were revealed in watermelon (Table 4). In the corresponding areas of the *A. thaliana* mitochondrial

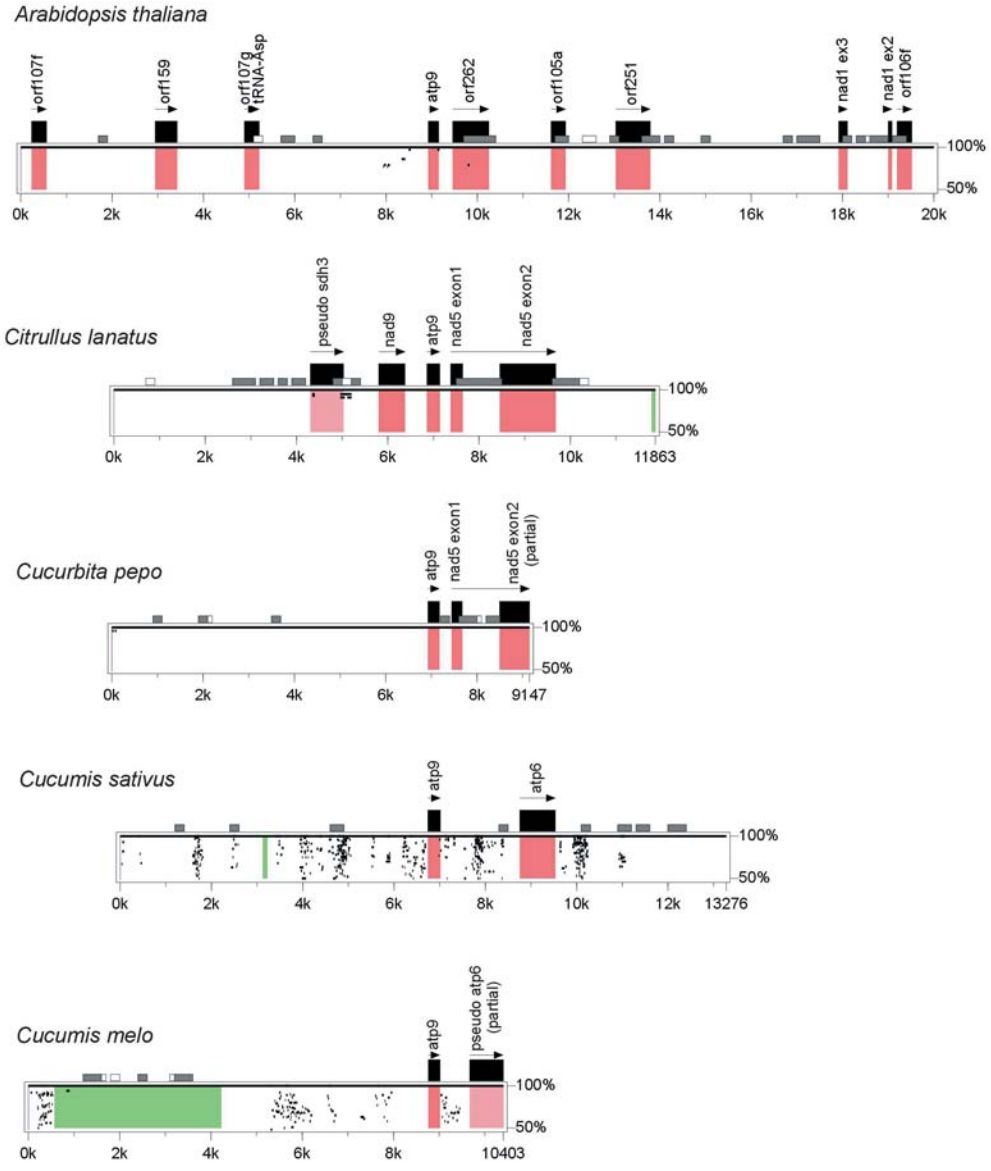

Fig. 4 *Dots* show locations of similar repetitive DNAs in the mitochondrial genomes of *A. thaliana*, watermelon, squash, cucumber and melon near the *atp9* coding regions as revealed by PipMaker (Schwartz et al. 2000). Genes are represented by *arrows and black boxes*. *Grey and white boxes* represent CpG/GpC rich areas (≥75% or ≥60%, respectively). Scale on the right side shows similarities from 50 to 100%

genome, six tandem repeats were revealed with copy numbers from 1.9 to 7.7. However, cucumber possessed many more dispersed direct and inverted repeats across similarly sized areas than the other cucurbits, *Arabidopsis* or sugar beet, and these sequences were concentrated in discrete pockets of repetitive DNAs (Table 5, Fig. 5). These inverted repeats were clearly a unique feature of the cucumber mitochondrial genome. Melon possessed three inverted repeats around both the *atp9* and *cob* genes, in contrast to 17 and 14 inverted repeats, respectively, in cucumber. The other cucurbits had no inverted repeats. *Arabidopsis* and sugar beet possess single inverted repeats in the genomic regions near the *atp9* and *cob* genes. Figure 5 shows the distribution of dispersed direct (A) and inverted (B) repeats in the cucumber *atp9* region. Some of these short repetitive motifs appeared as both direct and inverted repeats, possibly due to recombination events (Wolstenholme and Fauron 1995).

Table 5 Short dispersed direct and inverted repeats at minimum of 25 bp in the *Arabidopsis*, sugar beet and cucurbit mitochondrial genome near the *atp9* and *cob* coding regions

Plant	Direct repeats		Inverted repeats[1]	
	atp9	*cob*	*atp9*	*cob*
Watermelon	2	0	0	0
Squash	1	0	0	0
Cucumber	21	15	17	14
Melon	6	7	3	3
Arabidopsis	3	6	1	1
Sugar beet	1	1	0	1

[1] Repeats detected using Tandem Repeat Finder (Benson 1999) and REPuter software (Kurtz and Schleiermacher 1999)

The sequenced regions of the melon mitochondrial DNA had one major transfer from the chloroplast DNA (Fig. 4), accounting for 16% of the total sequenced regions. Therefore, based on the sequenced regions, the

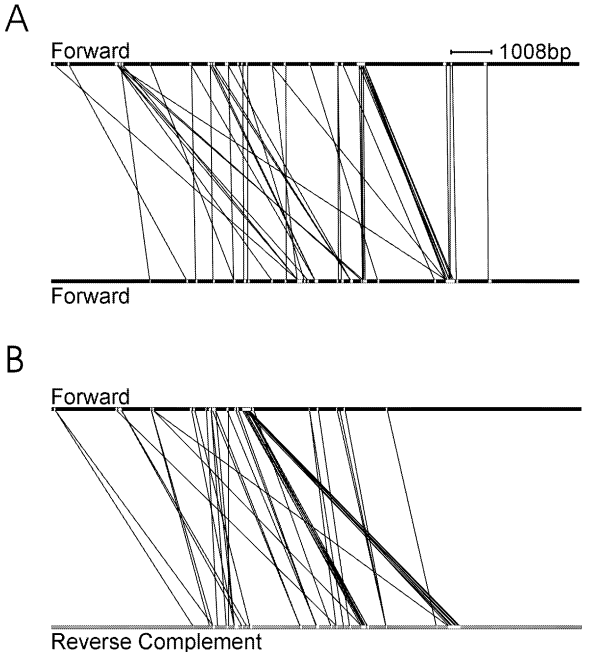

Fig. 5 Direct and inverted orientations of repetitive DNA sequences near *atp9* in the mitochondrial genome of cucumber as revealed by REPuter (Kurtz and Schleiermacher 1999)

accumulation of repetitive DNAs contributed more to the expansion of the cucumber mitochondrial DNA than to melon, indicating that different evolutionary forces may have contributed to the expansion of the mitochondrial genomes within the genus *Cucumis*.

Inverted-repeats in the cucumber mitochondrial DNA

Pipmaker revealed discrete pockets of direct and inverted DNA repeats on genomic contigs spanning the *atp9* and *cob* coding regions (Fig. 4). These localized concentrations of repetitive DNAs were strikingly similar to pockets of retroelements (Bennetzen 2000) or miniature inverted-repeat transposable elements (MITEs; Feschotte et al. 2002) in plant nuclear genomes. We evaluated the cucurbit mitochondrial sequences for retroviral elements. Two watermelon and melon *atp9* regions showed regions of short homologies to the *A. thaliana* copia-like retroelements, positions 4,789 to 5,198 bp (ATCOPIA32) in melon and positions 1,237 to 1,438 bp (ATCOPIA7I) in watermelon (Jurka 2000). The watermelon *atp9* region from 1,450 to 2,993 bp carried a degenerated transposon sequence showing significant similarity to the sugar-beet mitochondrial genome. We did not detect any homologies between the cucumber mitochondrial sequences and retroelements.

We built databases with mitochondrial sequences of the major cucurbits and the entire mitochondrial sequences of *A. thaliana* (Unseld et al. 1997) and sugar beet (Kubo et al. 2000), and used the program FINDMITE (Tu 2001) to reveal characteristics of the duplicated sequences. The sequenced regions of the melon mitochondrial genome (20.1 kb) possessed only four regions (0.2 per kb) with inverted repeats adjacent to short direct repeats (two with an inverted repeat of TCTTTGGTCCT and a direct repeat of AT, and one each with inverted repeats of CAAAGAATTTG and CAAAGAATTTG with direct repeats of AG and CG, respectively). *Arabidopsis* mitochondrial genome (366.9 kb) possessed only two such regions (0.005 per kb), both with an inverted repeat of ATAAAATGATT and the GC direct repeat. In contrast, 265.1 kb of the cucumber mitochondrial genome possessed 295 genomic regions with inverted repeats of at least 11 bp, short direct di- to tetranucleotide repeats, and lengths of 700 bp or less (1.1 per kb). The most common direct dinucleotide repeat was GC, which is in contrast to nuclear MITEs across a plethora of plants that show predominance of TA or AT direct repeats (Feschotte et al. 2002). The three most common inverted repeat sequences in cucumber were AT rich, such as AATATTCTTTG (24-times), GCAAAGAATAT(19-times), CAATATTCTTT (15-times) and TCTTTGCGCCT (14-times), which also differs from nuclear MITEs that tend to be more GC rich. We observed little to no internal conservation among sequences with the same inverted and direct repeats. Nuclear MITEs show internal sequence conservation of at least 85% over their entire length (Bureau et al. 1996). As a result, the cucumber repetitive regions showed little resemblance to bona-fide nuclear MITEs and most likely resulted from tandem duplication of mitochondrial regions carrying inverted sequences (Fig. 5).

Mitochondrial genome organization within *Cucumis*

Our sequence analyses of the mitochondrial genomes of cucumber, melon, squash and watermelon identified unique arrangements of coding regions. The *rpl5–rps14–cob* and *rrn5–rn18* operons are conserved in most plants (Aubert et al. 1992; Ye et al. 1993; Quiñones et al. 1996; Hoffmann et al. 1999), including squash and watermelon, but are rearranged in *Cucumis* (Fig. 4B). Rearrangements in the *Cucumis* mitochondrial genomes disrupted these transcriptional units and produced unique mitochondrial transcriptomes (Fig. 2). Our analyses also revealed that different forces may be responsible for the enormous expansion of the *Cucumis* mitochondrial DNAs. Short repetitive DNA motifs significantly contributed to the enlargement of the cucumber mitochondrial genome (Lilly and Havey 2001, Fig. 4). These discrete repetitive regions most likely correspond to the dispersed middle repetitive DNA fractions revealed by reassociation kinetics (Ward et al. 1981). Recombination among inverted repeats (Figs. 4 and 5) in these pockets of repetitive DNAs most likely shifted the arrangement of mitochondrial genes in cucumber and melon relative to watermelon and squash (Fig. 3, Fauron et al. 1995). Recombination among direct repeats (Fig. 5) would produce subgenomic circular DNA molecules coupled with duplication and deletion of specific sequences

between the repeats (Small et al. 1989; Fauron et al. 1990; Yamato et al. 1992; Albert et al. 1998). We demonstrated that a large mitochondrial deletion associated with the mosaic (MSC) phenotype of cucumber most likely resulted from multiple recombination events (Lilly et al. 2001; Bartoszewski et al., in press). Because large tracts of the *Cucumis* mitochondrial DNA have no obvious function, the deletion of specific regions, together with the duplication of another region, would occur without detriment to the plant (Albert et al. 1998). The mitochondrial genome of melon, although 35% larger than that of cucumber, possessed fewer concentrated regions of repetitive DNAs than cucumber (Fig. 4). Transfers from the chloroplast DNA were identified in melon, but not in cucumber mitochondrial DNA. We did not reveal specific repetitive or a duplicated sequence(s) significantly contributing to mitochondrial genome-expansion in both cucumber and melon. As a result, the accumulation of short repetitive DNAs contributed more to mitochondrial genome expansion in cucumber than melon, at least across the sequenced regions.

Acknowledgements We thank Vanessa Pope and Ruben Flores for help with sequence analyses, Dr. Cedric Feschotte (University of Georgia, USA) for critically reviewing our interpretation of MITE-like sequences, and Dr. Hanna Janska (University of Wroclaw, Poland) for critical reading of the manuscript. Grzegorz Bartoszewski was partially supported by the NATO Advanced Fellowship Programme. N. Katzir acknowledges the help of M. Kyle and S. McCouch during a research leave at Cornell University.

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